

β -Glycosphingolipids improve glucose intolerance and hepatic steatosis of the Cohen diabetic rat

Ehud Zigmond, Sarah W. Zangen, Orit Pappo, Miriam Sklair-Levy, Gadi Lalazar, Lydia Zolotaryova, Itamar Raz and Yaron Ilan

Am J Physiol Endocrinol Metab 296:E72-E78, 2009. First published 21 October 2008;
doi:10.1152/ajpendo.90634.2008

You might find this additional info useful...

Supplemental material for this article can be found at:

<http://ajpendo.physiology.org/content/suppl/2008/12/18/90634.2008.DC1.html>

This article cites 38 articles, 18 of which can be accessed free at:

<http://ajpendo.physiology.org/content/296/1/E72.full.html#ref-list-1>

Updated information and services including high resolution figures, can be found at:

<http://ajpendo.physiology.org/content/296/1/E72.full.html>

Additional material and information about *AJP - Endocrinology and Metabolism* can be found at:

<http://www.the-aps.org/publications/ajpendo>

This information is current as of September 20, 2011.

β -Glycosphingolipids improve glucose intolerance and hepatic steatosis of the Cohen diabetic rat

Ehud Zigmund,^{1*} Sarah W. Zangen,^{2*} Orit Pappo,³ Miriam Sklair-Levy,⁴ Gadi Lalazar,¹ Lydia Zolotaryova,¹ Itamar Raz,² and Yaron Ilan¹

¹Liver Unit and ²Diabetes Unit, Department of Internal Medicine, ³Department of Pathology, and ⁴Department of Radiology, Hebrew University-Hadassah Medical Center, Jerusalem, Israel

Submitted 28 July 2008; accepted in final form 7 October 2008

Zigmund E, Zangen SW, Pappo O, Sklair-Levy M, Lalazar G, Zolotaryova L, Raz I, Ilan Y. β -Glycosphingolipids improve glucose intolerance and hepatic steatosis of the Cohen diabetic rat. *Am J Physiol Endocrinol Metab* 296: E72–E78, 2009. First published October 21, 2008; doi:10.1152/ajpendo.90634.2008.—A link between altered levels of various gangliosides and the development of insulin resistance was described in transgenic mice. Naturally occurring glycosphingolipids were shown to exert immunomodulatory effects in a natural killer T (NKT) cell-dependent manner. This study examined whether glycosphingolipid-induced modulation of the immune system may reduce pancreatic and liver steatosis and stimulate insulin secretion in the Cohen diabetes-sensitive (CDS) rat, a lean model of non-insulin-resistant, nutritionally induced diabetes. Four groups of CDS rats fed a diabetogenic diet were treated with daily intraperitoneal injections of glycosphingolipids β -glucosylceramide, β -lactosylceramide, a combination of both (IGL), or vehicle (PBS) for up to 45 days. Immune modulation was assessed by fluorescence-activated cell sorting analysis of intrahepatic and intrasplenic lymphocytes. Steatosis was assessed by MRI imaging and histological examination of liver and pancreas. Blood glucose and plasma insulin concentrations were assessed during an oral glucose tolerance test. Administration of glycosphingolipids, particularly IGL, increased intrahepatic trapping of CD8 T and NKT lymphocytes. Pancreatic and liver histology were markedly improved and steatosis was reduced in all treated groups compared with vehicle-treated rats. Insulin secretion was restored after glycosphingolipid treatment, resulting in improved glucose tolerance. The immunomodulatory effect of β -glycosphingolipids improved the β -cell function of the hyperglycemic CDS rat. Thus our results suggest a role for the immune system in the pathogenesis of diabetes in this model.

glycolipids; immune response; natural killer T cells; type 2 diabetes

THE CHRONIC INCREASE in inflammatory mediators observed in type 2 diabetes (T2DM) has been shown to affect not only tissues and blood vessel walls but also pancreatic β -cells (11). The hyperglycemic Cohen diabetes-sensitive (CDS) rat is a model of mild cytokine-mediated diabetes (34). The CDS rat develops postprandial hyperglycemia when fed a diabetogenic high-sucrose, copper-poor diet (HSD), characterized by elevated blood glucose and blunted insulin secretion in response to glucose loading (8, 35, 36). Glucose intolerance in these rats is associated with exocrine lesions, lipid deposition, and IL-1 β -positive macrophage infiltration in the exocrine pancreas (34). However, the way in which the immune system is involved in the development of diabetes in this model has not yet been elucidated.

Natural killer T (NKT) cells, innate regulatory lymphocytes that express a conserved T-cell receptor, play important parts in diverse neoplastic, autoimmune, and infectious processes (3, 22). NKT cells usually express an invariant T-cell receptor that recognizes glycolipids in the context of the major histocompatibility complex (MHC) class I-like molecule CD1d (13, 15). β -Glucosylceramide (GC) and β -lactosylceramide (LC) are naturally occurring glycosphingolipids that exert immunomodulatory effects in an NKT cell-dependent manner (18, 20, 39).

CD8 and NKT lymphocytes playing a role in type 1 diabetes (T1DM) were recently found to be also important in the development of T2DM (26). β -Glycosphingolipids are NKT ligands, and their effects on CD8 cells may involve an indirect effect on dendritic cells (DCs) (3, 7, 33), affecting DC-CD8 or NKT-CD8 lymphocyte cross talk. The present study explores the possibility that by exerting an immunomodulatory effect synthetic β -glycosphingolipids can alleviate the metabolic derangements in the hyperglycemic CDS rat.

RESEARCH DESIGN AND METHODS

Animals. CDS rats were bred and maintained in the animal facility of Hebrew University School of Medicine, Jerusalem, Israel. Rats were fed a regular diet ad libitum (Koffolk, Petach-Tikva, Israel). The custom-prepared diabetogenic diet (HSD) contained 72% sucrose, 18% vitamin-free casein, 5% salt mixture no. II USP (MP Biomedicals), 4.5% butter, 0.5% corn oil, vitamins, and a low amount of copper (0.9 ppm) (8, 35). The protocol was approved by the Hebrew University Ethics Committee and complied with the Principles of Laboratory and Animal Regulations established by the National Society of Medical Research.

Glycolipids. GC and LC were purchased from Avanti Polar Lipids (Alabaster, AL). IGL is a 1:1 combination of GC and LC. Glycolipids were dissolved in ethanol and emulsified in phosphate-buffered saline (PBS).

Experimental group and study design. Eight-week-old male rats on a regular diet were divided into four treatment groups and switched to HSD. The rats were administered daily intraperitoneal injections of 2.5 mg/kg GC, LC, IGL, or PBS (control) for a total of 45 days. The effects of treatment on the immune system, steatosis, and liver and pancreatic damage were determined on days 30 and 45 of treatment.

Flow cytometry analysis for determination of CD4⁺, CD8⁺, and NKT lymphocyte subset distribution. At the end of the study (day 45), splenic and intrahepatic lymphocytes were isolated from rats in all experimental groups. Red blood cells were removed. The inferior vena cava was cut above the diaphragm, the spleen was removed, and the liver was flushed with 5 ml of cold PBS until it became pale. The liver was then placed in a 10-ml dish in cold, sterile PBS. Livers and

* E. Zigmund and S. W. Zangen contributed equally to this work.

Address for reprint requests and other correspondence: Y. Ilan, Liver Unit, Dept. of Medicine, Hebrew University-Hadassah Medical Center, PO Box 12000, Jerusalem, Israel IL-91120 (e-mail: ilan@hadassah.org.il).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

spleens were crushed through a stainless steel mesh (size 60, Sigma Chemical, St. Louis, MO). Cell suspensions were placed in a 50-ml tube for 3 min and washed twice in cold PBS (1,250 rpm for 10 min), and debris was removed. Cells were resuspended in PBS, the cell suspension was passed through a nylon mesh presoaked in PBS, and unbound cells were collected in a 50-ml tube. Cells were washed twice in 45 ml of PBS (1,250 rpm at room temperature) and suspended in 7 ml of PBS. For liver and spleen lymphocyte isolation, 20 ml of Histopaque-1077 (Sigma Diagnostics, St. Louis, MO) was slowly placed underneath the 7 ml of cells in the 50-ml tube. The tube was centrifuged at 1,640 rpm for 15 min at room temperature. Cells at the interface were collected, diluted in a 50-ml tube, and washed twice with ice-cold PBS (1,250 rpm for 10 min). Approximately 1×10^6 cells/spleen liver were recovered. By Trypan blue staining, cell viability was estimated as >95%.

Immediately after lymphocyte isolation, triplicates of 2.5×10^4 cells/500 μ l PBS were placed into Falcon 2052 tubes, incubated with 4 ml of 1% BSA for 10 min, and centrifuged at 1,400 rpm for 5 min. Cells were resuspended in 10 μ l of FCS. For analysis of the different subsets of T lymphocytes, anti-CD3 antibodies were combined with anti-NK1.1, anti-CD4, or anti-CD8 antibodies (Pharmingen). Analytical cell sorting was performed on 1×10^5 cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson, Oxnard, CA). Data were analyzed with the Consort 30 two-color contour plot program (Becton Dickinson) and with the CELLQuest 25 program.

Evaluation of effect of β -glycosphingolipids on blood lipid levels. The effect of β -glycosphingolipid treatment on lipid levels was assessed by measurement of serum cholesterol, free fatty acid, and triglyceride levels in blood by standard techniques at day 43, the end of the study period.

Evaluation of effect of β -glycosphingolipids on glucose and insulin levels. Rats underwent an oral glucose tolerance test (OGTT) with the following protocol. Blood glucose and plasma insulin concentrations were measured after overnight fast (0) and at 15, 30, 60, 90, and 120 min after the oral administration of glucose (3.5 g/kg), as described previously (34). Glucose concentration in tail blood was measured with a standard glucometer (Elite, Bayer, Leverkusen, Germany). Plasma insulin level was determined with an ultrasensitive rat insulin ELISA assay (Mercodia, Uppsala, Sweden). Graphs in Fig. 6 present the results of the OGTT at 30 days of diet and treatment. Similar data were obtained at day 43 (data not shown).

Evaluation of effect of β -glycosphingolipids on pancreatic damage. At the end of the study, the pancreases of rats from the four treatment groups were removed and their weight was recorded. Paraffin-embedded pancreatic sections (5 μ m) stained with hematoxylin and eosin were evaluated for islet morphology, pancreatic steatosis, and acinar degeneration. The effect of β -glycosphingolipid treatment on the degree of pancreatic damage was assessed by an experienced pathologist in a blinded fashion. Degrees of hepatic and pancreatic steatosis were classified according to the following scores: grade 0, no steatosis; grade 1, 5% steatosis; grade 2, 10% steatosis; grade 3, >15% steatosis. Sections were obtained from each rat in all groups.

Evaluation of effect of β -glycosphingolipids on hepatic steatosis. The degree of hepatic steatosis was assessed by MRI imaging on day 28. Hepatic and pancreatic fat contents were measured by a T1-weighted dual-echo chemical shift gradient-echo sequence technique that provides in-phase and out-of-phase images in a single acquisition for assessment and quantification of fat. T1-weighted in-/out-of-phase MRI is sufficiently sensitive for the detection of relatively small proportions of tissue fat (23, 24). MR images were acquired by a 1.5-T system (Sigma LX; GE, Milwaukee, WI). Dual-echo MRI was performed with a repetition time (TR) of 125 ms, dual echo times (TEs) of 4 and 6.5 ms, and a flip angle of 80°. Imaging parameters included section thickness of 3 mm, a 13-cm field of view, and a 256×160 matrix. Axial and coronal images were obtained. Signal intensity (SI) changes between in-phase and out-of-phase images were computed.

The SI index was calculated as follows: SI index(2) = $(SI_{in} - SI_{out})/SI_{in}$, where SI_{in} is in-phase SI and SI_{out} is out-of-phase SI. Low SI index values indicate a smaller amount of tissue fat.

Evaluation of effect of β -glycosphingolipids on liver damage. Assessment of liver damage was performed by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in peripheral blood by standard techniques at the end of the study. Paraffin-embedded liver sections (5 μ m) stained with hematoxylin and eosin and Oil Red O (frozen sections) were evaluated by an experienced pathologist in a blinded fashion. Sections were scored by using steatosis and inflammatory scores as described previously (5). The percentage of involved area per field was calculated for each of the five liver sections from each rat in all groups.

Evaluation of effect of β -glycosphingolipids on serum transforming growth factor- β levels. The effect of β -glycosphingolipid treatment on serum transforming growth factor (TGF)- β levels was measured by "sandwich" ELISA with a commercial kit (Genzyme Diagnostics) according to the manufacturer's instructions.

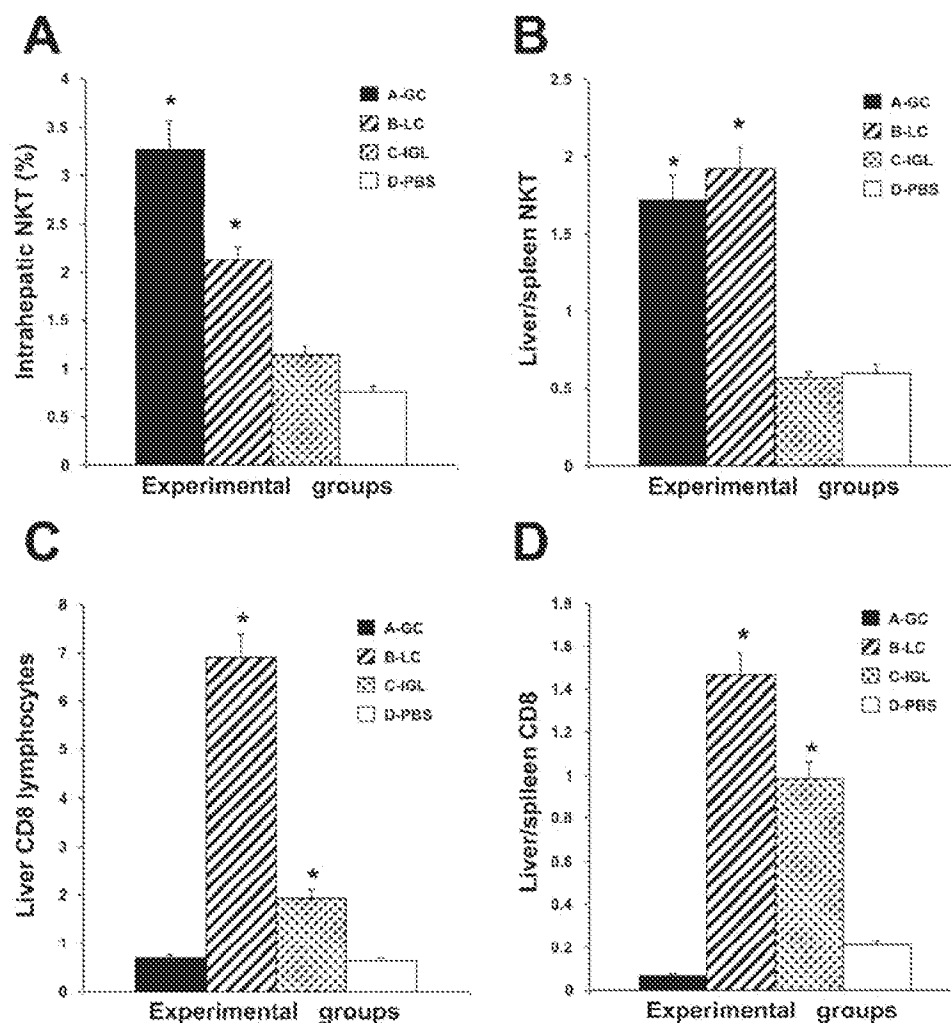
Statistical analysis. Data shown are means \pm SE. Statistical significance of differences between groups was determined by one-way ANOVA followed by the Tukey test with the Sigmaplot program (Jandel, San Rafael, CA). A two-tailed paired *t*-test was used to compare data from tests performed on the same animal. A *P* value <0.05 was considered significant.

RESULTS

Effect of β -glycosphingolipids on intrahepatic CD8 and NKT lymphocyte distributions. We have determined the effect of β -glycosphingolipids on the distribution of CD8 and NKT lymphocytes. Administration of β -glycosphingolipids increased the percentage of intrahepatic NKT cell trapping in all of the treated groups compared with the PBS-treated group ($P < 0.005$) (Fig. 1A). However, the percentages of intrasplenic NKT cells increased only in GC (1.9 ± 0.07) and IGL (2.0 ± 0.26)-treated groups compared with PBS-treated rats ($1.25 \pm 0.15\%$). The calculated ratio of intrahepatic to intrasplenic NKT lymphocytes revealed that LC- and GC-treated groups manifested significant increases in intrahepatic NKT lymphocytes compared with the control PBS-treated group ($P = 0.002$, Fig. 1B). The percentages of intrahepatic CD8 lymphocytes were elevated ($P = 0.003$) in LC- and IGL-treated rats compared with PBS-treated rats ($0.64 \pm 0.065\%$, Fig. 1C). The calculated ratio of intrahepatic to intrasplenic CD8 lymphocytes revealed that LC- and IGL-treated groups manifested significant increases in intrahepatic CD8 lymphocytes compared with the control PBS-treated group ($P = 0.002$, Fig. 1D). No significant effects were noted on CD4 lymphocytes (data not shown).

Effect of β -glycosphingolipids on pancreatic steatosis and weight. We have determined the effect of β -glycosphingolipids on the pancreas. Decrease in pancreatic weight was shown to be highly associated with diabetes development in the CDS rat (34). We therefore assessed whether treatment with β -glycosphingolipids may halt the decrease in pancreatic weight. Pancreas weight was significantly higher in rats treated with GC, LC, or IGL compared with the control PBS group ($P < 0.005$, Fig. 2A). A marked decrease in pancreatic steatosis score was noted in GC- and IGL-treated animals vs. PBS ($P < 0.05$, Fig. 2B). The exocrine pancreas of the GC- and IGL-treated groups had a normal appearance compared with the PBS group, in which substantial fat infiltration, atrophy, and fibrotic characteristics were detected (Fig. 2C). The increase in pancreatic weight was associated with substantial reductions in

Fig. 1. Effect of β -glycosphingolipids on intrahepatic CD8 and natural killer T (NKT) lymphocyte distribution. Fluorescence-activated cell sorting analysis of lymphocytes isolated from livers and spleens of Cohen diabetes-sensitive (CDS) rats fed high-sucrose diet (HSD) and treated with a daily intraperitoneal injection of phosphate-buffered saline (PBS; control, $n = 10$) or 2.5 mg/kg glycosphingolipids [glucosylceramide (GC), $n = 7$; lactosylceramide (LC), $n = 10$; or GC + LC (IGL), $n = 10$] for 1 mo. CD4⁺, CD8⁺, and NKT cells were sorted. A: % of intrahepatic NKT cells after the different treatments. B: ratio of intrahepatic to intrasplenic NKT lymphocytes in each experimental group. C: % of intrahepatic CD8 cells after the different treatments. D: ratio of intrahepatic to intrasplenic CD8 lymphocytes in each experimental group. * $P < 0.005$.



pancreatic damage in all glycosphingolipid-treated groups. No alteration in body weight was noted for any treatment group (data not shown).

Effect of β -glycosphingolipids on serum lipid levels. A significant reduction in serum triglycerides ($P < 0.05$) and cholesterol ($P = 0.002$) was observed in IGL-treated rats compared with PBS-treated rats (Fig. 3).

Effect of β -glycosphingolipids on liver damage. A reduction in MRI hepatic steatosis index indicative of reduced fat content was observed in rats treated with IGL compared with PBS-treated rats ($P < 0.05$; Fig. 4). Hepatic damage was also reduced after LC and IGL, as revealed by the reduced serum levels of the hepatic transaminases compared with PBS ($P < 0.03$; Fig. 5A). Examination of liver histology revealed improvements in the microscopic scores for LC- and IGL-treated animals compared with the PBS-treated hyperglycemic CDS rat. The percentages of involved area per field were $10.0 \pm 1.7\%$ and $11.7 \pm 1.5\%$ vs. $18.5 \pm 2.3\%$ for LC and IGL vs. PBS, respectively ($P < 0.03$). IGL administration produced the most pronounced effect on liver histology. The mean microscopic score for IGL was reduced compared with PBS ($P < 0.005$; Fig. 5B).

Effects of β -glycosphingolipids on glucose and insulin levels. Administration of β -glycosphingolipids improved glucose-stimulated insulin secretion (GSIS) as assessed by OGTT.

Insulin area under the curve (AUC) increased markedly in GC ($P < 0.001$ vs. PBS)- and IGL ($P < 0.04$)-treated rats compared with PBS (Fig. 6A; Supplemental Data).¹ Treatment with β -glycosphingolipids significantly reduced the glucose AUC in all treated groups ($P < 0.02$ vs. PBS-treated rats (Fig. 6B)).

Effect of β -glycosphingolipids on serum TGF- β levels. Administration of GC, LC, and IGL was associated with a significant increase in serum TGF- β levels compared with PBS ($P < 0.05$; Fig. 7).

DISCUSSION

In the present study, we investigated the effect of administration of β -glycosphingolipids on glucose intolerance of the hyperglycemic CDS rat, a model of mild cytokine-mediated diabetic syndrome that does not exhibit peripheral resistance to insulin. We explored the possibility that by exerting an immunomodulatory effect, β -glycosphingolipids may improve the β -cell function of the hyperglycemic CDS rat. The data presented in this study demonstrate improved glucose tolerance and increased GSIS after β -glycosphingolipid administration. Interestingly, the improvement in β -cell function vs. PBS was highly associated with an increased intrahepatic trapping of

¹ The online version of this article contains supplemental material.

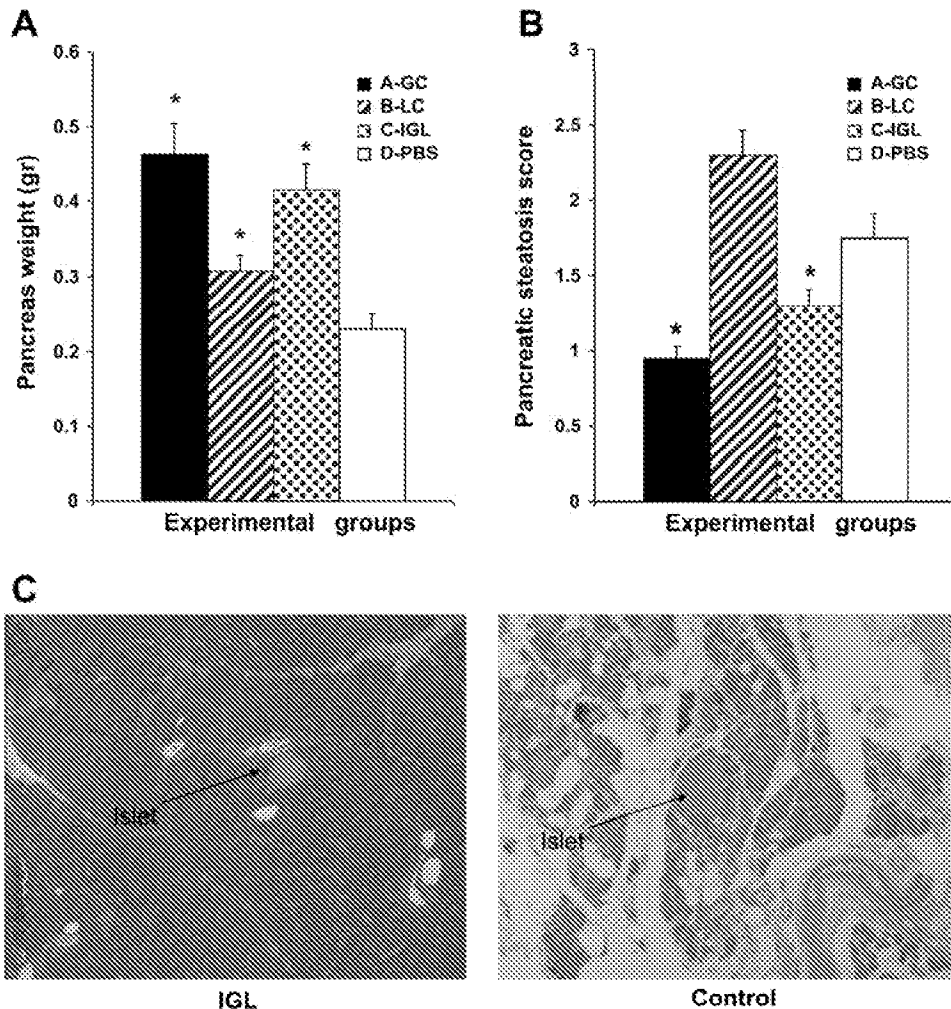


Fig. 2. Effect of β -glycosphingolipids on pancreatic damage. *A*: pancreatic weight of CDS rats fed HSD and treated with a daily intraperitoneal injection of PBS (control, $n = 10$) or 2.5 mg/kg glycosphingolipids (GC, $n = 10$; LC, $n = 10$; or IGL, $n = 10$) for 1 mo. *B*: hematoxylin and eosin staining of pancreatic sections from PBS- and IGL-treated CDS rats fed HSD for 1 mo. The pancreatic steatosis score was calculated from the scoring of 5 pancreatic sections per rat. Decreased degrees of steatosis were noted in GC- and IGL-treated animals. *C*: pancreatic sections of IGL-treated rats showed significantly decreased steatosis; islets were not affected (arrow). *D*: pancreatic sections from PBS-treated rats exhibited depletion of the acini in the exocrine pancreas and fat infiltration in the tissue; islets were not affected (arrow). * $P < 0.005$.

CD8 and NKT lymphocytes, which may imply a role of the innate immune system in the development of diabetes in this rat model.

The involvement of the immune system was recently reported in hyperglycemic CDS rats, in which the development of diabetes was associated with infiltration of activated macrophages into the damaged exocrine pancreas (34). The findings of the present study reinforce the possible involvement of the immune system in diabetes development in this rat model and suggest that modulation of the immune system may improve glucose metabolism. Modulation of the immune system was shown to improve glucose metabolism in both human patients and animal models of diabetes. In patients with T2DM, exercise-induced increase in CD4⁺CD25⁺ regulatory T cells (Tregs; T lymphocytes) correlated with decreased hemoglobin A1C levels (37), while CD4⁺CD25⁺ Tregs were shown to modulate the activity of the insulin receptor in an animal model of T2DM (37). In T1DM, an NKT lymphocyte subset was suggested to play a key regulatory role in linking the innate and adaptive immune systems (3). Activation of invariant NKT (iNKT) cells by the administration of β -galactosylceramide was shown to protect nonobese diabetic (NOD) mice from T1DM development (14). Protection was enabled because of induction and accumulation of the effector cells (9, 27).

CD4⁺CD25⁺ Tregs maintained self-tolerance and prevented autoimmune attack on the β -cells of these mice (19, 26).

NKT cell responses are induced by recognition of glycolipid antigens presented by CD1d, an antigen presentation protein (3). In the last 10 years great strides have been made in understanding the types of glycolipids recognized by NKT cells. A number of ligands presented by CD1d or other CD1d-restricted cells to the NKT lymphocytes (6) are involved in prevention of several immune-mediated disorders. In this study, the β -glycosphingolipids GC, LC, and IGL, which are natural intermediates in the metabolic pathways of ceramides (18), were studied because they have been shown to exert NKT-mediated immunomodulatory effects in several other models (39). Administration of GC in vivo attenuates NKT-mediated damage in animal models of diabetes, concanavalin A hepatitis, and immune-mediated colitis (20, 21, 39). Preliminary data suggest that GC may play a similar role in humans with the metabolic syndrome by affecting NKT cell distribution (38). In our study, the glycosphingolipids were shown to affect several metabolic parameters. The greatest effect on most of the parameters studied was achieved with IGL, a combination of two different glycosphingolipids, while when given separately GC and LC had beneficial effects on different aspects of the metabolic syndrome. The disparities among

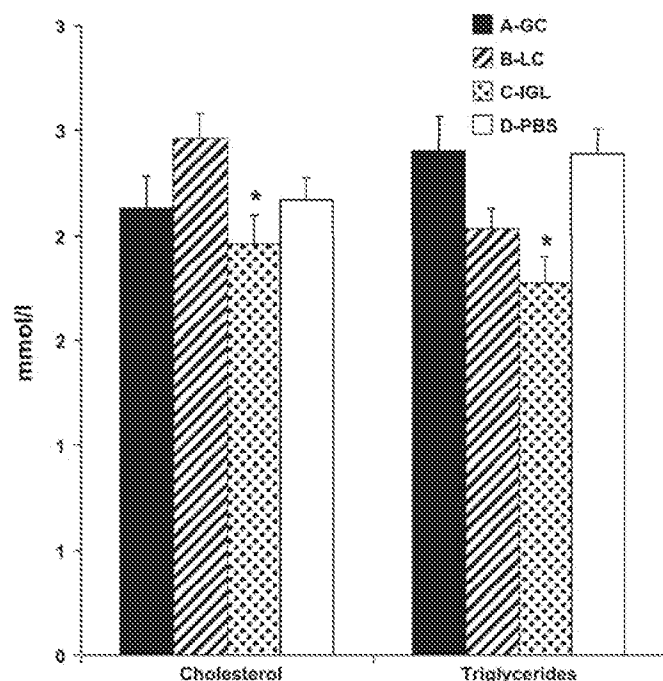


Fig. 3. Effect of β -glycosphingolipids on lipid profile. Serum triglyceride and cholesterol levels of overnight-fasted CDS rats fed HSD and treated with a daily intraperitoneal injection of PBS (control, $n = 10$) or 2.5 mg/kg glycosphingolipids (GC, $n = 10$; LC, $n = 9$; or IGL, $n = 10$) for 1 mo. * $P < 0.005$.

different β -glycosphingolipids and a relative advantage of IGL over the other ligands may be explained by the higher affinity of the CD1d receptor on the NKT cells to a specific glycosphingolipid or to the combination of two different glycosphingolipids (28–30). In addition, a ligand-associated dendritic cell effect might also account for these differences (12). Alternatively, it may be suggested that since the glycosphingolipids affect multiple tissues that are involved in glucose homeostasis, including the liver, pancreas, and adipose tissue, the out-

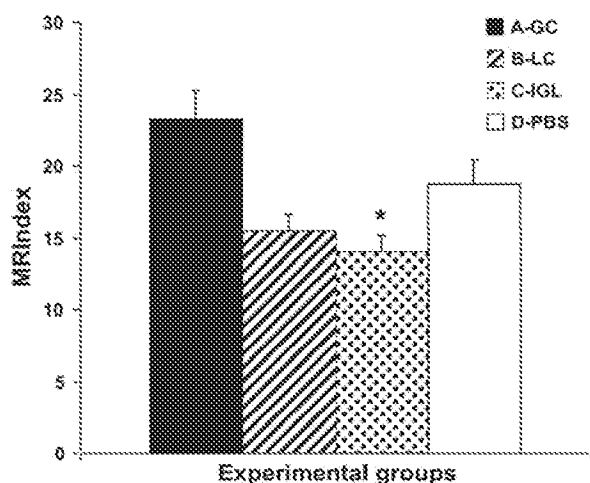


Fig. 4. Effect of β -glycosphingolipids on liver steatosis. MRI quantification of liver fat performed on CDS rats fed HSD and treated with a daily intraperitoneal injection of PBS (control, $n = 10$) or 2.5 mg/kg glycosphingolipids (GC, $n = 10$; LC, $n = 9$; or IGL, $n = 10$). MRI was performed after 28 days of treatment.

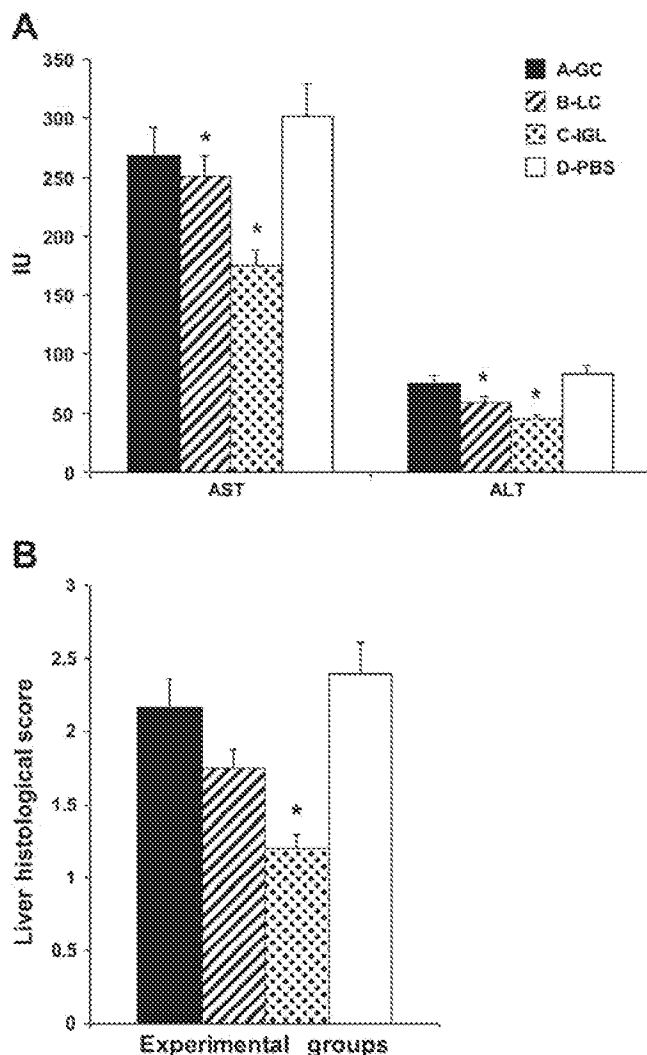


Fig. 5. Effect of β -glycosphingolipids on liver damage. A: effect on liver enzymes. Liver enzymes in the sera of overnight-fasted CDS rats fed HSD were measured after 45 days of intraperitoneal injections of PBS (control, $n = 10$) or 2.5 mg/kg glycosphingolipids (GC, $n = 10$; LC, $n = 9$; or IGL, $n = 10$). AST, aspartate aminotransferase; ALT, alanine aminotransferase. B: effect on liver histology. Five liver sections from CDS rats fed HSD were scored (according to score index described in RESEARCH DESIGN AND METHODS) for inflammation and steatosis after 45 days of intraperitoneal injections of PBS (control, $n = 10$) or 2.5 mg/kg glycosphingolipids (GC, $n = 10$; LC, $n = 9$; or IGL, $n = 10$). * $P < 0.005$.

come of their effect is dependent on the characteristics of the target used.

The role of glycosphingolipids in diabetes development is controversial and is greatly related to the identity of the glycosphingolipid administered and the characteristics of the animal model studied. In our study administration of the β -glycosphingolipids resulted in improved glucose metabolism, while in Zucker diabetic fatty rats reduction in blood glucose levels was achieved by lowering glucosphingolipid levels with a glucosylceramide synthase inhibitor (1, 37). The discrepancy between the studies may be explained by a difference in the rat models used in the studies. Diabetes in Zucker diabetic fatty rats is related to peripheral resistance. Since glycosphingolipids were shown to negatively modulate the activity of the insulin receptor (31), the use of Genz-123346, an inhibitor of glycosphin-

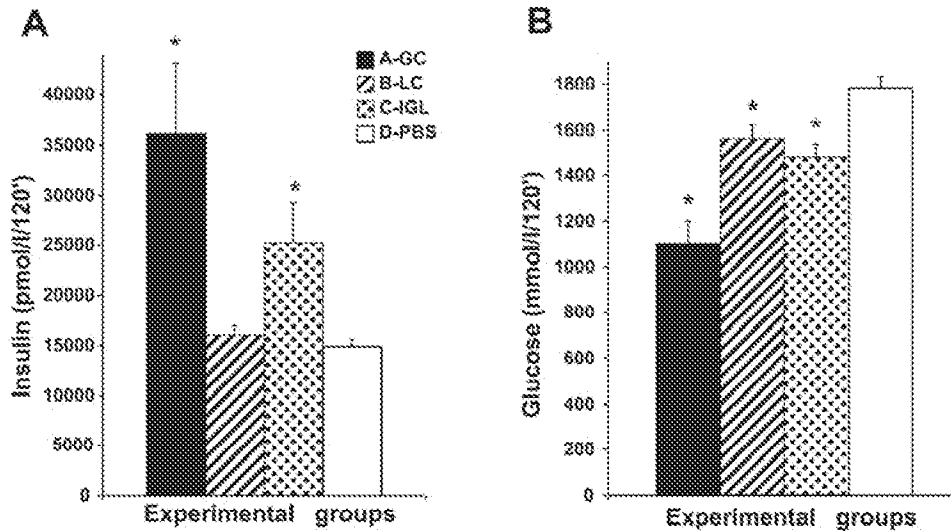


Fig. 6. Effect of β -glycosphingolipids on oral glucose tolerance test (OGTT). Area under the curve is shown for insulin (A) and glucose (B) after OGTT (glucose, 3.5 g/kg) performed on CDS rats fed HSD and treated with a daily intraperitoneal injection of PBS (control, $n = 16$) or 2.5 mg/kg glycosphingolipids (GC, $n = 7$; LC, $n = 9$; or IGL, $n = 10$) for 1 mo. * $P < 0.005$.

golipid synthesis, is likely to improve insulin sensitivity and glucose control and increase in Zucker diabetic fatty rats. Furthermore, the inhibitor Genz-123346 did not alter insulin secretion in these rats, suggesting that its effect is mainly due to increase in peripheral insulin sensitivity. In contrast, the hyperglycemic CDS rat does not exhibit peripheral insulin resistance, or obesity and diabetes development is solely attributed to β -cell dysfunction. We may therefore argue that in our study elevation of β -glycosphingolipid levels affected β -cell function with no or only a minor influence on peripheral insulin resistance. Thus additional experiments are needed to determine the mechanism underlying the different effects of the glycosphingolipids on the metabolic syndrome.

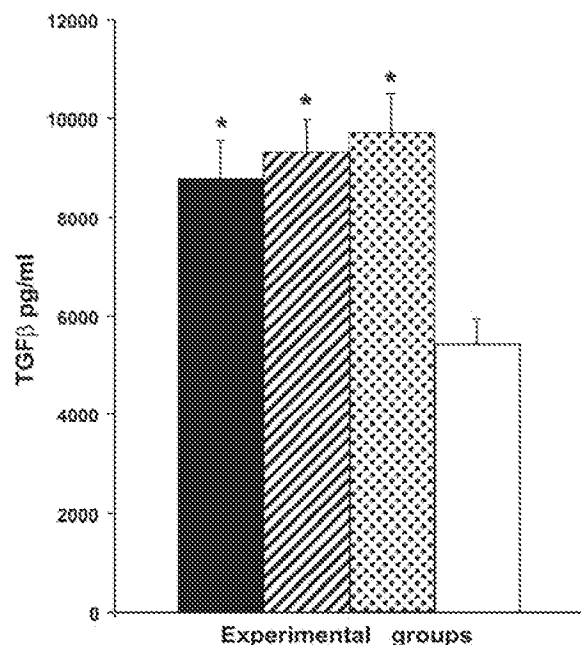


Fig. 7. Effect of β -glycosphingolipids on serum transforming growth factor (TGF)- β levels. Serum TGF- β levels were measured from CDS rats fed HSD after 45 days of intraperitoneal injections of PBS (control, $n = 16$) or 2.5 mg/kg glycosphingolipids (GC, $n = 10$; LC, $n = 9$; or IGL, $n = 10$). * $P < 0.005$.

The beneficial effect of β -glycosphingolipids on β -cell function seems to be mediated via an immunomodulatory effect on CD8 or NKT cells. The improvement in insulin secretion was accompanied by a decrease in pancreatic and liver damage and by a reduction in lipid infiltration and local inflammation parallel with increased levels of serum TGF- β levels. The differences on NKT cells noted between GC and IGL may be related to different effects on DCs affecting the CD1d-dependent response of NKT cells (16, 17).

The present study also supports a role for a chronic inflammatory response, specifically a CD8- and/or NKT-dependent mechanism, in the pathogenesis of diabetes in the CDS rat model. In recent years, chronic inflammation was suggested to be an important pathophysiological mechanism in T2DM (4, 10, 11, 32) using both insulin resistance and pancreatic β -cell dysfunction. In the process of inflammation macrophages and the endothelium were shown to contribute to the increased serum levels of different cytokines including IL-1 β , IL-6, and TNF in T2DM patients (25). The increase in the levels of these cytokines may induce the liver to produce acute-phase proteins, which could impair β -cell secretory function. Thus, alternatively, our data could suggest that the primary effect of the β -glycosphingolipids is on the inflammatory pathways, while their effect on the immune system itself is a by-product of this effect.

Finally, our data do not rule out a contributing effect of other subsets of Tregs that were not measured in the present study. Future ongoing studies using additional glycosphingolipids and studying several parameters related to the inflammatory process will provide better understanding of the mechanisms underlying the effect of the glycosphingolipids in β -cell function. However, although the reports from the different studies may be contradictory, all studies are in agreement that glycosphingolipid synthesis is a potentially important and underexploited pathway that may provide new therapeutic targets to treat diabetes.

In summary, diabetes development in the CDS rat is coupled with exocrine damage, fat infiltration, and local inflammation. Treatment with β -glycosphingolipids improved β -cell function and induced a marked improvement in hepatic and exocrine damage. We suggest that the β -glycosphingolipids through

their immunomodulatory qualities inhibit local inflammation in the target organ, resulting in alleviation of pancreatic and liver damage and in the subsequent restoration of β-cell function.

GRANTS

This work was supported by the Russell Berrie Foundation and D-Cure, Diabetes Care in Israel, grants from the A. M. Cohen Foundation for the Advancement of Research of the Cohen Diabetic Rat to S. W. Zangen, the Rouman-Epstein Liver Research Foundation, and ENZO Biochem, New York, NY.

REFERENCES

- Aerts JM, Ottenhoff R, Powlson AS, Grefhorst A, van Eijk M, Dubbelhuis PF, Aten J, Kuipers F, Serlie MJ, Wennekes T, Sethi JK, O'Rahilly S, Overkleeft HS. Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity. *Diabetes* 56: 1341–1349, 2007.
- Agrati C, Nisli C, Oliva A, D'Offizi G, Montesano C, Pocillo LP, Poccia F. Lymphocyte distribution and intrahepatic compartmentalization during HCV infection: a main role for MHC-unrestricted T cells. *Arch Immunol Ther Exp (Warsz)* 50: 307–316, 2002.
- Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* 25: 297–336, 2007.
- Bloomgarden ZT. Inflammation, atherosclerosis, and aspects of insulin action. *Diabetes Care* 28: 2312–2319, 2005.
- Brunt EM. Nonalcoholic steatohepatitis: definition and pathology. *Semin Liver Dis* 21: 3–16, 2001.
- Brutkiewicz RR. CD1d ligands: the good, the bad, and the ugly. *J Immunol* 177: 769–775, 2006.
- Buzas EI, Gyorgy B, Pasztoi M, Jelinek I, Falus A, Gabius HJ. Carbohydrate recognition systems in autoimmunity. *Autoimmunity* 39: 691–704, 2006.
- Cohen AM. Development of the model. In: *The Cohen Diabetic Rat*, edited by Cohen A and Rosenmann E. Basel: Karger, 1990, p. 1–9.
- Crispe IN. Hepatic T cells and liver tolerance. *Nat Rev Immunol* 3: 51–62, 2003.
- Donath MY, Halban PA. Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. *Diabetologia* 47: 581–589, 2004.
- Donath MY, Storch J, Maedler K, Mandrup-Poulsen T. Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. *J Mol Med* 81: 455–470, 2003.
- Fujii S, Shimizu K, Hemmi H, Steinman RM. Invariant Valpha14⁺ natural killer T cells mature dendritic cells, leading to strong adaptive immunity. *Immunol Rev* 220: 183–198, 2007.
- Gumperz JE. CD1d-restricted "NKT" cells and myeloid IL-12 production: an immunological crossroads leading to promotion or suppression of effective anti-tumor immune responses? *J Leukoc Biol* 76: 307–313, 2004.
- Hussain S, Wagner M, Ly D, Delovitch TL. Role of regulatory invariant CD1d-restricted natural killer T cells in protection against type 1 diabetes. *Immunol Res* 31: 177–188, 2005.
- Kawano T, Cui J, Kozuka Y, Taura I, Kaneko Y, Motoki K, Deno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278: 1626–1629, 1997.
- Lalazar G, Ben Ya'acov A, Eliakim-Raz N, Livovsky DM, Pappo O, Preston S, Zolotarov L, Ilan Y. Beta-glycosphingolipids-mediated lipid raft alteration is associated with redistribution of NKT cells and increased intrahepatic CD8⁺ T lymphocyte trapping. *J Lipid Res* 49: 1884–1893, 2008.
- Lalazar G, Ben Ya'acov A, Lador A, Livovsky DM, Pappo O, Preston S, Harezi M, Ilan Y. Modulation of intracellular machinery by beta-glycolipids is associated with alteration of NKT lipid rafts and amelioration of concanavalin-induced hepatitis. *Mol Immunol* 45: 3517–3525, 2008.
- Lalazar G, Preston S, Zigmund E, Ben Ya'acov A, Ilan Y. Glycolipids as immune modulatory tools. *Mini Rev Med Chem* 6: 1249–1253, 2006.
- Ly D, Mi QS, Hussain S, Delovitch TL. Protection from type 1 diabetes by invariant NK T cells requires the activity of CD4⁺CD25⁺ regulatory T cells. *J Immunol* 177: 3695–3704, 2006.
- Margalit M, Ghazala SA, Alper R, Elinav E, Klein A, Doviner V, Sherman Y, Thalenfeld B, Engelhardt D, Rabbani E, Ilan Y. Glucocerebroside treatment ameliorates ConA hepatitis by inhibition of NKT lymphocytes. *Am J Physiol Gastrointest Liver Physiol* 289: G917–G925, 2005.
- Margalit M, Shalev Z, Pappo O, Sklar-Levy M, Alper R, Gomori M, Engelhardt D, Rabbani E, Ilan Y. Glucocerebroside ameliorates the metabolic syndrome in OB/OS mice. *J Pharmacol Exp Ther* 319: 105–110, 2006.
- Mars LT, Novak J, Liblau RS, Lehen A. Therapeutic manipulation of iNKT cells in autoimmunity: modes of action and potential risks. *Trends Immunol* 25: 471–476, 2004.
- Mitchell DG, Kim I, Chang TS, Vinitski S, Consigny PM, Saponaro SA, Ehrlich SM, Rifkin MD, Rubin R. Fatty liver. Chemical shift phase-difference and suppression magnetic resonance imaging techniques in animals, phantoms, and humans. *Invest Radiol* 26: 1041–1052, 1991.
- Namimoto T, Yamashita Y, Mitsuzaki K, Nakayama Y, Makita O, Kadota M, Takahashi M. Adrenal masses: quantification of fat content with double-echo chemical shift in-phase and opposed-phase FLASH MR images for differentiation of adrenal adenomas. *Radiology* 218: 642–646, 2001.
- Pickup JC, Crook MA. Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia* 41: 1241–1248, 1998.
- Sharif S, Arreaza GA, Zucker P, Delovitch TL. Regulatory natural killer T cells protect against spontaneous and recurrent type 1 diabetes. *Ann NY Acad Sci* 958: 77–88, 2002.
- Shibolet O, Alper R, Zolotarov L, Trop S, Thalenfeld B, Engelhardt D, Rabbani E, Ilan Y. The role of intrahepatic CD8⁺ T cell trapping and NK1.1⁺ cells in liver-mediated immune regulation. *Clin Immunol* 111: 82–92, 2004.
- Stanic AK, De Silva AD, Park JJ, Sriram V, Ichikawa S, Hirabayashi Y, Hayakawa K, Van Kaer L, Brutkiewicz RR, Joyce S. Defective presentation of the CD1d1-restricted natural Valpha14⁺ NKT lymphocyte antigen caused by beta-D-glucosylceramide synthase deficiency. *Proc Natl Acad Sci USA* 100: 1849–1854, 2003.
- Stanic AK, Park JJ, Joyce S. Invariant self recognition by an invariant, rearranged T-cell receptor and its immune consequences. *Immunology* 109: 171–184, 2003.
- Stanic AK, Shashidharamurthy R, Bezbradica JS, Matsuki N, Yoshimura Y, Miyake S, Choi EY, Schell TD, Van Kaer L, Tevethia SS, Roopenian DC, Yamamura T, Joyce S. Another view of T cell antigen recognition: cooperative engagement of glycolipid antigens by Valpha14⁺ T(NKT) cell receptor. *J Immunol* 171: 4539–4551, 2003.
- Tagami S, Inokuchi J, Kabayama K, Yoshimura H, Kitamura E, Iemura S, Ogawa C, Ishii A, Saito M, Ohtsuka Y, Sakane S, Igarashi Y. Ganglioside GM3 participates in the pathological conditions of insulin resistance. *J Biol Chem* 277: 3085–3092, 2002.
- Tan KC, Chow WS, Tam S, Bucala R, Betteridge J. Association between acute-phase reactants and advanced glycation end products in type 2 diabetes. *Diabetes Care* 27: 223–228, 2004.
- Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol* 5: 405–417, 2007.
- Weksler-Zangen S, Raz I, Leuzen S, Jorns A, Ehrenfeld S, Amir G, Oprea A, Yagil Y, Yagil C, Zangen DH, Kaiser N. Impaired glucose stimulated insulin secretion is coupled with exocrine pancreatic lesions in the Cohen diabetic rat. *Diabetes* 57: 279–287, 2008.
- Weksler-Zangen S, Yagil C, Zangen DH, Ornoy A, Jacob HJ, Yagil Y. The newly inbred Cohen diabetic rat: a nonobese normolipidemic genetic model of diet-induced type 2 diabetes expressing sex differences. *Diabetes* 50: 2521–2529, 2001.
- Yagil C, Barak A, Ben-Dor D, Rosenmann E, Bernheim J, Rosner M, Segov Y, Weksler-Zangen S, Raz I, Yagil Y. Nonproteinuric diabetes-associated nephropathy in the Cohen rat model of type 2 diabetes. *Diabetes* 54: 1487–1496, 2005.
- Zhao H, Przybylska M, Wu H, Zhang J, Siegel C, Komarnitsky S, Yew NS, Cheng SH. Inhibiting glycosphingolipid synthesis improves glycemic control and insulin sensitivity in animal models of type 2 diabetes. *Diabetes* 56: 1210–1218, 2007.
- Zigmund E, Pappo O, Zangen S, Levy Sklar M, Hemed N, Rabbani E, Itamar R, Ilan Y, Margalit M. Treatment of non-alcoholic steatohepatitis by β-glucosylceramide: a phase I/II clinical study (Abstract). *Hepatology* 44: 180A, 2006.
- Zigmund E, Preston S, Pappo O, Lalazar G, Margalit M, Shalev Z, Zolotarov L, Friedman D, Alper R, Ilan Y. Beta-glucosylceramide: a novel method for enhancement of natural killer T lymphocyte plasticity in murine models of immune-mediated disorders. *Gut* 56: 82–89, 2007.